PB86-243250

Biochemical and Neuropathological Assessment of Triphenyl Phosphite in Rats

(U.S.) Health Effects Research Lab.
Research Triangle Park, NC

1986

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1. REPORT NO. EPA/600/J-86/123	2. PB86-243250
4. TITLE AND SUBTITLE BIOCHEMICAL AND NEUROPATHOLOGICAL ASSESSMENT OF TRIPHENYL PHOSPHITE IN RATS (Journal Version)	5. REPORT DATE 1986 6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S) Bellina Veronesi[1], Stephanie Padilla[1], and Dale Newland[2]	8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS [1]U.S. EPA, Health Effects Research Laboratory, RTP, NC, 27711 [2] Northrop Services, Inc. Environmental Science Laboratory, RTP, NC 27710	10. PROGRAM ELEMENT NO. 11. CONTRACT/GRANT NO.
12. SPONSORING AGENCY NAME AND ADDRESS Health Effects Research Laboratory Office of Research and Development U.S. Environmental Protection Agency Research Triangle Park, N.C. 27711	13. TYPE OF REPORT AND PERIOD COVERED Journal Article 14. SPONSORING AGENCY CODE EPA-600/11

15. SUPPLEMENTARY NOTES
Citation: Toxicology and Applied Pharmacology, Volume 83, No. 2, April 1986

16. ABSTRACT

The putative neurotoxicity of the organophosphorus compound triphenyl phosphite (TPP) was examined in Long Evans, adult male rats. Animals were exposed to two 1.0 ml/kg (1184 mg/kg) injections (sc) of TPP spaced 1 week apart and sampled for biochemical and neuropathological examination. At the time of sampling, rats displayed dysfunctional changes including tail rigidity, circling, and hindlimb paralysis. Neuropathic damage was confined to the lateral and ventral columns of all spinal levels and consisted of myelin ellipsoids and giant axonal swellings filled with smooth endoplasmic reticulum. Wallerian-like degeneration was observed in the spinal roots, the sciatic nerve, and tibial branches. Biochemical assessment of brain acetylcholinesterase (AChE) and neurotoxic esterase (NTE) activity was determined 1, 4, 24, 48, and 72 hr after the second TPP treatment. Both enzyme activity concentrations were depressed maximally at 48 hr postexposure by 30 and 39%, respectively. Serum cholinesterase, sampled 48 hr after the second TPP exposure was depressed by 33%. Data from this study indicate that subchronic exposure to the organophosphite TPP results in severe neurotoxic consequences which differ from those previously described in rats with organophosphorus-induced delayed neuropathy.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group

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18. DISTRIBUTION STATEMENT RELEASE TO PUBLIC	19. SECURITY CLASS *(This Report)* UNCLASSIFIED	21. NO. OF PAGES 11
	20. SECURITY CLASS *(This page)* UNCLASSIFIED	22. PRICE

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TOXICOLOGY AND APPLIED PHARMACOLOGY 83, 203–210 (1986)

EPA/600/J-86/123
JOURNAL ARTICLE

Biochemical and Neuropathological Assessment of Triphenyl Phosphite in Rats

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Received July 27, 1985; accepted November 6, 1985

Biochemical and Neuropathological Assessment of Triphenyl Phosphite in Rats. VERONESI, B., PADILLA, S., AND NEWLAND, D. (1986). *Toxicol. Appl. Pharmacol.* **83**, 203–210. The putative neurotoxicity of the organophosphorus compound triphenyl phosphite (TPP) was examined in Long Evans, adult male rats. Animals were exposed to two 1.0 ml/kg (1184 mg/kg) injections (sc) of TPP spaced 1 week apart and sampled for biochemical and neuropathological examination. At the time of sampling, rats displayed dysfunctional changes including tail rigidity, circling, and hindlimb paralysis. Neuropathic damage was confined to the lateral and ventral columns of all spinal levels and consisted of myelin ellipsoids and giant axonal swellings filled with smooth endoplasmic reticulum. Wallerian-like degeneration was observed in the spinal roots, the sciatic nerve, and tibial branches. Biochemical assessment of brain acetylcholinesterase (AChE) and neurotoxic esterase (NTE) activity was determined 1, 4, 24, 48, and 72 hr after the second TPP treatment. Both enzyme activity concentrations were depressed maximally at 48 hr postexposure by 30 and 39%, respectively. Serum cholinesterase, sampled 48 hr after the second TPP exposure was depressed by 33%. Data from this study indicate that subchronic exposure to the organophosphite TPP results in severe neurotoxic consequences which differ from those previously described in rats with organophosphorus-induced delayed neuropathy.

The organophosphorus (OP) compound, triphenyl phosphite (TPP), is widely used in industry as a rubber and plastic stabilizer, a metal scavenger, and a diluent of epoxy resins (U.S. EPA, 1985). TPP is also used in agriculture to synthesize herbicides (i.e., [*n*-(*O,O*-diphenylphosphonomethyl glycine ethyl ester)], Kuwahara *et al.*, 1977), as an insecticide synergist (Hemingway and Georghiou, 1984), and as an anti-fungal foliar agent (Rusuku *et al.*, 1984). The volume statistics for TPP indicate that in 1977 between 10 and 51 million pounds were produced or imported into the United States (U.S. EPA, 1977). Smith and colleagues (1933) first alluded to the neurotoxicity of TPP in early studies on the phenol esters, reporting severe debilitation in cats exposed to acute or mutiple doses of the aryl phosphite. More recent studies, compiled by the Huntingdon Research Centre in England (Roberts *et al.*, 1982), reported that dermal administration of TPP to the combs of hens resulted in paralysis, serum cholinesterase inhibition, and spinal cord and peripheral nerve histopathology. In light of these data, which are characteristic of organophosphorus-induced delayed neuropathy (OPIDN) (Abou-Donia, 1981), and because of TPP's familial resemblance to model OPIDN-compounds, notably tri-*ortho*-cresyl phosphate (TOCP) (Fig. 1), its putative neurotoxicity was exam-

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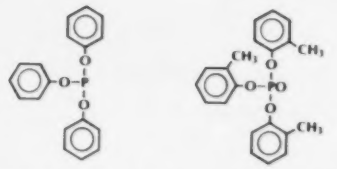


Triphenyl phosphite (TPP) Tri-ortho-cresyl phosphate (TOCP)

FIG. 1. The familial resemblance of the model OPIDN compound TOCP and TPP is shown. Both organophosphorus compounds contain a reactive phosphorus group and hydrolyzable ester bonds.

ined in rats, using morphological and biochemical end-points that are pathognomonic of OPIDN (Veronesi, 1984; Padilla and Veronesi, 1985).

METHODS

Chemicals. Technical grade triphenyl phosphite (TPP) was obtained commercially (ICN Co., Plainview, N.Y., Lot 19575) and analyzed by gas chromatography/mass spectrometry (GC/MS) by a commercial laboratory (Triangle Laboratories, Inc., Research Triangle Park, N.C.) to determine impurities. Paraoxon (*O,O*-diethyl *O*,4-nitrophenylphosphate, ≥92% pure) was obtained from Aldrich Chemical Company (Milwaukee, Wisc.). Mipafox (*N,N'*-diisopropylphosphorodiamidic fluoride; ≥98% pure) and phenyl valerate (≥99% pure) were synthesized by Dr. S. Soliman (Alexandria, Egypt). All other chemicals were from Sigma Chemical Company (St. Louis, Mo.) or Fisher Scientific Company (Pittsburgh, Pa.).

TPP analysis. Combined GC/MS analysis were performed using a VG-Masslab (Model 12-250) quadrupole GC/MS system. Sample introduction was by means of Grob-type splitless injection on to a 30 m DB-1 column (J and W Scientific, Rancho Cordova, Calif.) which was connected directly to the ion source of the mass spectrometer. Mass spectra were obtained by scanning from a mass-to-charge ratio of 35 to 510 in 0.95 sec with a 0.05-sec interscan delay. Experimental spectra were compared to a 25,000 spectrum library. Peak areas were used to estimate percentage composition for the TPP sample. The response factor was determined for phenol, while equal response factors were assumed for TPP and triphenyl phosphate.

Animals. Male Long Evans rats, 60 to 90 days old (Charles River, Portage, Me.) were housed in pairs on sterile bedding. Animals were fed *ad libitum* with Purina Rat Chow and allowed free access to water. Animal rooms were kept on a 12-hr alternating light/dark cycle and held at 22°C and 50% humidity. Rats were exposed to two 1.0 ml/kg (1184 mg/kg) sc injections of TPP spaced 1 week apart. Control animals received an equivalent volume of saline. Because of TPP's reported anti-cholinesterase properties (Smith *et al.*, 1933; Roberts *et al.*, 1982), prophylactic atropine sulfate treatments (15 mg/kg) were administered (ip) to all animals 20 min pre- and 4 hr post-exposure to TPP or saline. Each animal's functional impairments were evaluated at sampling time. Because of the phenol impurity noted in our TPP sample, an additional group of animals were dosed (sc) with two injections of phenol (200 mg/kg) spaced 1 week apart.

Neuropathology: Tissue Preparation. Rats treated with TPP (n = 10), phenol (n = 6), and saline (n = 10) were sampled 14 days after initial exposure. Animals were anesthetized with Nembutal (40 mg/kg) containing 1% heparin (50 units/ml) prior to intracardiac perfusion. A cannula was inserted through the ventricle and the body flushed with phosphate-buffered 4% paraformaldehyde, followed by Sorenson's buffered 5% glutaraldehyde. The spinal cord at three levels, i.e., cervical (C2), lumbar (L2–5), and sacral (S1–4), was sampled. Peripheral nerve (PNS) fibers examined included the dorsal and ventral spinal roots at the lower cord levels, the tibial branches, and the sciatic nerve at the notch. PNS fibers were examined in both cross and longitudinal sections. All tissues were postfixed in Dalton's 2% osmium tetroxide for 1 hr, and then dehydrated in increasing grades of ethanol, cleared in propylene oxide, and embedded in Polybed 812 (Polysciences, Inc., Warrington, Pa.). Semi-thin (1.0 μm) sections were cut with glass knives and stained with 1% toluidine blue-o in 1% sodium borate. Areas of interest were photographed with an Olympus Vannox microscope using Kodak Technical Pan Film 2415 (ASA 100). Thin (60 to 70 nm) sections were cut with a diamond knife, stained with uranyl acetate and lead citrate, and photographed with a Phillips 410 electron microscope.

NTE assay. Equal numbers (n = 3 to 8) of TPP-treated and control animals were decapitated at 1, 4, 24, 48, and 72 hr after the last treatment. Neurotoxic esterase (NTE), the putative target enzyme of OPIDN (Johnson, 1969, 1970), was assayed in the following manner. The brain (including the cerebellum) was removed, weighed, and immediately placed in 5 vol (w/v) of ice-cold buffer (50 mM Tris/HCl, 0.2 mM EDTA, pH 8.0). After homogenization at 4°C for 15 sec using a polytron apparatus (Brinkman Instruments, Westbury, N.Y.), the homogenate was centrifuged (4°C) at 9000g for 15 min. The brain 9K supernatant fraction, rather than the more turbid tissue homogenate, was used for enzyme analysis. NTE activity was assayed on the day of termination according to the method of Johnson (1977) with some modification (Soliman *et al.*, 1982). NTE activity was defined as the difference in phenyl valerate hydrolyzing activity between paired samples in the presence of either paraoxon or paraoxon plus Mipafox (Johnson, 1977; Soliman *et al.*, 1982). Protein concentrations were determined according to the method of Lowry *et al.* (1951) with bovine serum albumin

(fraction V) as the standard. Phenol-treated rats ($n = 9$) and saline controls ($n = 3$) were killed 48 hr after the last phenol treatment for brain NTE determination.

Cholinesterase assay. Brain acetylcholinesterase (AChE) was assayed radiometrically (Johnson and Russell, 1975). Eighty microliters of the 9K supernatant fraction was incubated for 1 min with 20 μl of 5 μM acetylcholine iodide. (0.1 μCi [^{3}H]acetylcholine iodide/tube (New England Nuclear, Boston, Mass.; 5 mCi/mmol). At the end of the incubation period, the reaction was stopped, 5 ml of the scintillation mixture added, and the samples were counted within 24 hr on a Beckman LS 7000 scintillation counter with a counting efficiency of 20%.

Erythrocyte (EC 3.1.1.7) and serum cholinesterase (EC 3.1.1.8) activities were determined spectrophotometrically (Ellman *et al.*, 1961) by a contract laboratory (Northrop Services Inc., Research Triangle Park, N.C.) in a CentrifiChem automated analyzer. Blood samples, obtained by heart puncture, were submitted from TPP-treated ($n = 5$) and control ($n = 5$) animals 48 hr after the last exposure.

Statistics. NTE or AChE activity in treated animals was compared to the control values at each time point using an F test for variance ratio followed by the appropriate Student's t test for significant differences. Percentage inhibition was calculated by dividing the enzyme activity value for each treated animal by the mean activity value for their respective control group.

RESULTS

TPP Analysis

GC/MS analysis of the TPP sample indicated three components sufficiently volatile to elute from the GC column. The components gave a mass spectrum consistent with TPP (93.9%), phenol (0.8%), and triphenyl phosphate (5.3%) (Fig. 2).

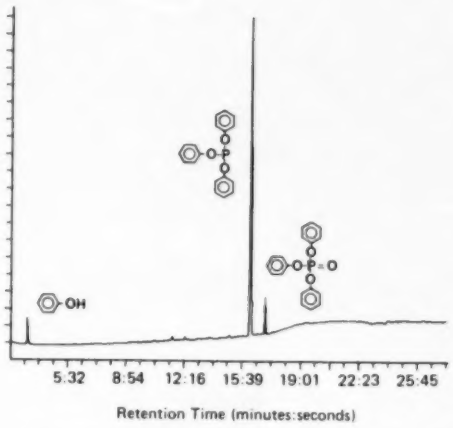


FIG. 2. Gas chromatographic column tracing of the TPP sample. The structure as determined by mass spectrometry analysis is indicated adjacent to each peak.

Animal Condition

Immediately after exposure, TPP-treated rats displayed generalized tremors which subsided after a few hours. Although no mortality occurred, animals appeared ruffled and generally lethargic. Edema, skin ulcerations, and necrosis occurred at the injection site of approximately 25% of the animals. Phenol-treated rats experienced transient tonic convulsions 10 to 15 min postdosing but recovered after 1 to 2 hr. At the time of sampling, approximately 50% of the TPP-exposed rats displayed dysfunctional changes characterized by tail rigidity, circling behavior, and hindlimb paralysis. When placed on their backs, the more severely affected rats would remain recumbent or roll into a ball, in contrast to control animals which would roll over quickly into an upright position. Phenol-treated rats appeared normal at the time of termination.

Microscopy

Examination of 1 μm spinal cord sections from TPP-treated rats revealed massive, swollen axons, and multiple myelin ellipsoids in the lateral and ventral columns of the sacral, lumbar, and cervical cord. These giant axonal swellings were located frequently in the intermediate gray zone and adjacent to the ventral gray horn. In the cervical cord, degeneration was seen in the dorsolateral fasciculus (Lissauer's zone) in the ventral–lateral and ventral columns, and throughout the area prefissuralis (Fig. 3). When examined by electron microscopy, the axonal lesions contained masses of interlaced smooth endoplasmic reticulum and

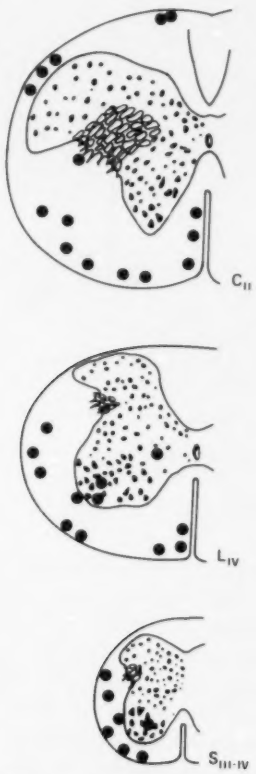


FIG. 3. A diagram of the topography of neuropathic damage in TPP-treated rats is shown. The location of axonal swellings and myelin ellipsoids (dots) was largely confined to the lateral and ventral columns of all spinal cord regions.

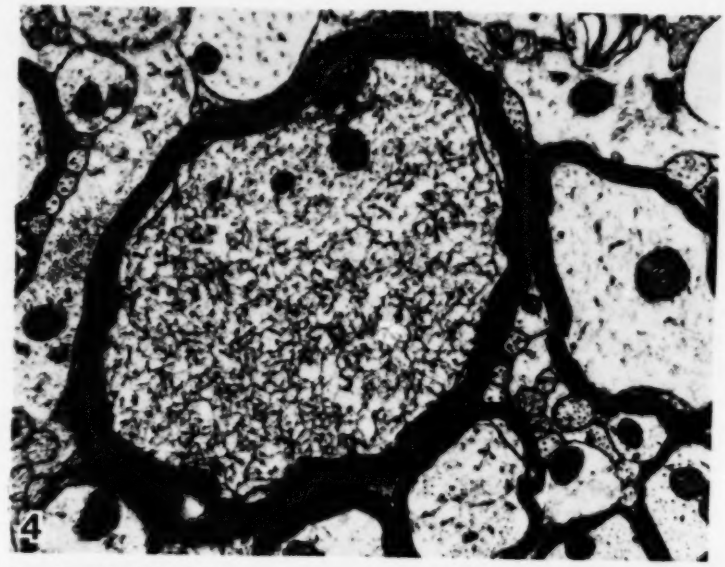
channels of aggregated mitochondria and neurofilaments (Fig. 4). In animals that displayed ataxia and circling, damage was bilateral and involved all cord levels. In the least affected animals, however, damage was confined to the sacral and lumbar cord with minimal involvement of the cervical cord's lateral–ventral columns. Evidence of anterior horn cell necrosis was recorded in the sacral and lumbar cord (Rex laminae IX) of severely dysfunctional rats (Fig. 5). Wallerian-like degeneration, consisting of myelin fragments, fiber debris, and macrophage infiltration, was noted in the lumbar and sacral spinal roots, in the sciatic nerve and the tibial branches of TPP-treated animals (Fig. 6). In two phenol-treated rats, spinal cord and spinal root degeneration was recorded. The neuropathic data are summarized in Table 1.

NTE and AChE Activity

Brain NTE and AChE activity data for the TPP-treated rats were assembled over a 72-hr period (Fig. 7). NTE was significantly inhibited ($p \leq 0.05$) at 4 and 24 hr postdosing. Maximum inhibition (38.9%; $p \leq 0.005$) of brain NTE activity in the TPP-treated animals occurred by 48 hr, but returned to control levels by 72 hr. The only significant ($p \leq 0.005$) inhibition of brain AChE was by 30% at 48 hr postdosing. Although erythrocyte cholinesterase was unaffected at the 48 hr sample time (Controls: 100 ± 4.52 mU/ml; TPP-treated: 100.2 ± 2.87 mU/ml), serum cholinesterase activity was significantly depressed by 33% (Controls 434.8 ± 34.26 mU/ml; TPP-treated: 288.0 ± 19.74 mU/ml). No brain NTE inhibition was noted in the phenol-treated rats 48 hr after the last phenol administration.

DISCUSSION

This study reports that subchronic exposure to the organophosphite TPP produces spinal cord damage and ataxia in the rat, yet only marginally (≤39%) inhibits NTE, the putative target enzyme of OPIDN (Johnson, 1969, 1970). These data differ from the neurotoxic profile of OPIDN previously described in the rat (Padilla and Veronesi, 1985; Veronesi *et al.*, 1986) and the chicken (Johnson, 1974; Ohkawa *et al.*, 1980), which mandates an inhibition of mean brain NTE activity by ≥65% for the development of the delayed neuropathy associated with neurotoxic OPs. In view of the close correlation between NTE inhibition and OPIDN that has been previously established for hundreds of tested compounds, these results raise questions as to the proper nosology of TPP neuropathy. Inspection of the pattern



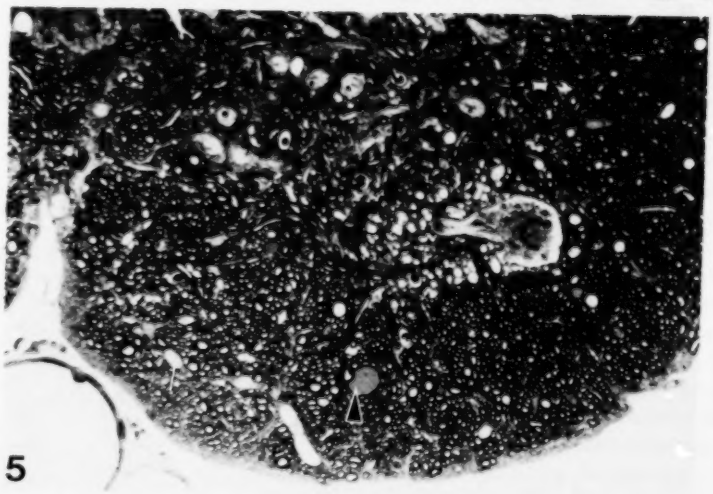


FIG. 4. TPP-induced axonal swellings contained interlaced agranular endoplasmic reticulum and channels of neurofilaments and mitochondria. ×17,050.

FIG. 5. Anterior horn cell necrosis was occassionally observed in the lower spinal cord levels in combination with giant axonal swellings (arrow). The spinal cord histology is otherwise unremarkable. ×1341.

of TPP-induced spinal cord damage suggests that it differs from that seen in the conventional (e.g., chicken, cat) models of OPIDN, where degeneration initiates in and preferentially damages the large diameter ascending and descending spinal tracts, concurrently (Cavanagh, 1954, 1964). A similar pattern of neuropathic degeneration has been recently described in rats exposed to the neurotoxic organophosphates, TOCP and Mipafox (Vero-

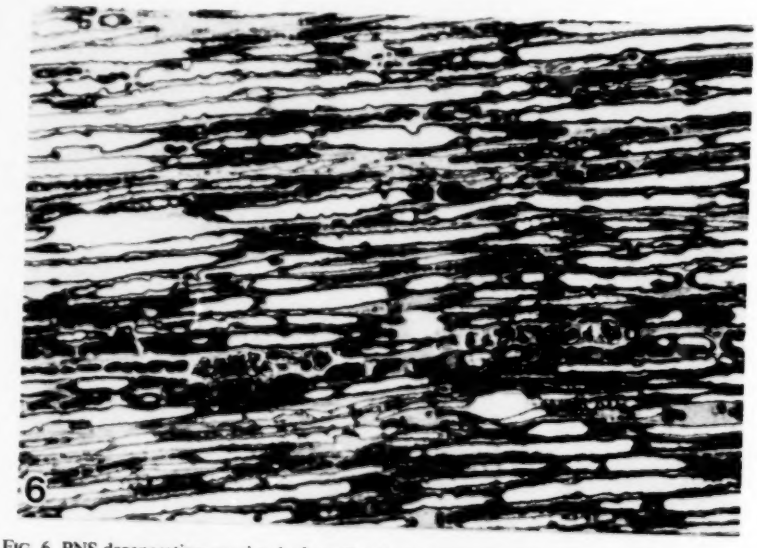


FIG. 6. PNS degeneration consisted of myelin sheath fragments and axonal ballooning. ×1261.

nesi, 1984; Veronesi and Padilla, 1985). In the rodent model of OPIDN, the cervical cord's fasciculus gracilis is the earliest and most severely damaged tract and only after repeated exposures are the lateral and ventral columns of the cervical cord involved. In contrast, the most vulnerable spinal tracts in TPP neuropathy are those located in the lateral and ventral columns of all cord levels, with a conspicuous sparing of the large diameter ascending tracts of the dorsal columns. Although a definitive description of TPP's topography of neuropathic damage requires examination of additional levels of the neuraxis, the predilection of damage to those columns which house the rubrospinal, tectospinal, and vestibulospinal efferents and the spinothalamic and spinocerebellar afferents (Zemlan, *et al.*, 1978, 1979; Giesler, *et al.*, 1981) may underlie the dysfunctions which characterize this neuropathy. The circling behavior and tail rigidity are peculiar to OPIDN, but have been described in other toxic neuropathies, notably that produced by β,β-iminodipropionitrile (Chou and Hartmann, 1964) and suggests a supraspinal involvement, a possibility presently under investigation.

NTE inhibition does not appear to play a critical role in the pathogenesis of TPP neuropathy in the rat. Although TPP's molecular

TABLE 1

TPP-NEUROPATHIC DAMAGE

	TPP	Phenol	Saline
Dysfunction			
Ataxia	6/10[a]	0/6	0/10
Circling	5/10	0/6	0/10
Tail rigidity	5/10	0/6	0/10
Spinal cord			
Cervical	6/10	0/6	0/10
Lumbar	9/10	0/6	0/10
Sacral	10/10	2/6	0/10
Spinal roots			
Dorsal	6/10	2/6	0/10
Ventral	3/10	1/6	0/10
PNS			
Sciatic	8/10	0/6	0/10
Tibial branches	9/10	0/6	0/10

[a] Fraction represents the number of animals per test group that showed unequivocal neuropathic or dysfunctional change. In the cord this was defined histologically as the presence of >2 axonal swellings or myelin ellipsoids per section. In the PNS this was defined as >3 myelin ellipsoids in combination with macrophages.

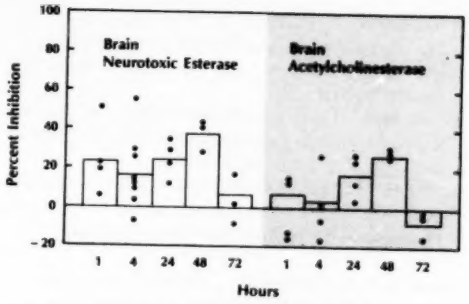


FIG. 7. NTE and AChE inhibition in TPP-treated rats assayed at 1 to 72 hr after the second TPP administration. Bar heights represent the mean values for each group while the filled circles represent values from individual animals. Control values ($\bar{x} \pm$ SE) for NTE were: 1 hr: 8.59 ± 0.32; 4 hr: 8.72 ± 0.54; 24 hr: 9.14 ± 0.49; 48 hr: 10.22 ± 0.37; 72 hr: 8.50 ± 0.42 nmol phenyl valerate hydrolyzed/min/mg protein and for AChE were: 1 hr: 25313 ± 968; 4 hr: 18082 ± 1378; 24 hr: 21623 ± 1398; 48 hr: 22716 ± 968; 72 hr: 17612 ± 762 cpm/mg Lowry protein.

structure contains both a reactive phosphorus and hydrolyzable ester bonds, suggesting that it should readily inhibit and "age" NTE (Clothier and Johnson, 1980), these same bonds render the aryl phosphite highly unstable and reactive to other enzymes (Eto, 1974) whose inhibition may be more culpable to the neuropathic damage noted in this study. Early studies (Aird *et al.*, 1940) emphasized the hydrolyzability of TPP to phenol, an impurity noted in our test lot of TPP. Although spinal root degeneration has been related to topographically applied phenol (Smith, 1964; Schaumburg *et al.*, 1970), our data indicate a neglible neuropathic effect when phenol is systemically administered. GC/MS analysis also determined triphenyl phosphate to be a TPP contaminant. This compound, formed by the oxidation of the phosphite, had been originally labeled neuropathic (Smith *et al.*, 1933; Dollahite and Pierce, 1969). Later investigations (Wills *et al.*, 1979), however, instigated by pure (99.8%) triphenyl phosphate's inability to inhibit NTE (Johnson, 1975), discredited its neurotoxicity.

In summary, this study indicates that subchronic exposure to the aryl phosphite TPP, produces in rats dysfunctional changes and central (spinal cord) and peripheral neuropathy. The pattern of neuropathic damage, however, in conjunction with marginal NTE inhibition, suggests that this toxic neuropathy does not relate to previous descriptions of OPIDN in the rat, and demonstrates a caveat to using a biochemical assay in the rat as a sole predictor of OPIDN.

ACKNOWLEDGMENTS

The authors thank Al Inman, Donald Lyerly, and Tom Grizzle for their excellent technical assistance. The research described in this article has been reviewed by the Health Effects Research Laboratory, U.S. Environmental Protection Agency and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the Agency nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

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